REMARKS

1. Disposition of Claims

Claims 2, 8, and 16-20 are pending in this application. Claims 1, 3-7, 9-15, and 21-24 have been canceled as being drawn to non-elected subject matter. No new matter has been added. Reexamination and reconsideration of the application, as amended, are respectfully requested.

2. Compliance With 35 USC 103

As an initial matter, the rejections of record have been withdrawn in favor of new rejections. The issue is whether claims 2, 8, and 17 are in compliance with 35 USC 103 or unpatentable as being obvious over Westaway et al. (USP 6,893,866, see also WO 99/28487 of which the USP is a continuation application), and/or Khromykh & Westaway, J. Virol. 71: 1497 (1997), in view of Polo et al., J. Virol. 71: 5366 (1997).

USP 6,893,866 described construction of subgenomic replicons of flavivirus Kunjin and their packaging into virus-like particles by a packaging cell line. Khromykh & Westaway 1997 published construction of subgenomic replicons of flavivirus Kunjin. Polo et al. 1997 described the preparation of a stable full-length cDNA copy of flavivirus dengue virus type 2 RNA capable of producing an infectious RNA transcript in vitro.

The Patent Office takes the position that Khromykh & Westaway 1997 described extending Khromykh & Westaway, J. Virol. 68: 4580 (1994) (attached) to prepare a Kunjin self-replicating RNA (subgenomic replicon) with a deletion in the region of the genome encoding structural components of the virion. Khromykh & Westaway 1994 published the preparation of a stable full-length cDNA copy of Kunjin RNA capable of producing an infectious RNA transcript in vitro. Thus, reasoned the Patent Office, it would have been obvious to extend Polo et al. 1997 publishing the preparation of a stable full-length cDNA copy of dengue virus type 2 RNA capable of producing an infectious RNA transcript in vitro to prepare a dengue virus type 2 self-replicating RNA (subgenomic replicon) with a deletion in the region of the genome encoding structural components of the virion.

As it turns out, the prior art actually teaches away from the claimed subgenomic replicons.[1] The rule according to MPEP 2145 is that teaching away is a significant factor to be considered in determining obviousness, where the prior art criticizes, discredits, or otherwise discourages the solution claimed. Under MPEP 2145 X D 3, proceeding contrary to accepted wisdom is evidence of nonobviousness.

According to Polo et al. 1997, p. 5366, col. 2, first full paragraph, two-piece construction had been necessary in the case of dengue virus type 2 because full-length cDNA clones capable of producing infectious transcripts were never obtained. To use these two-clone systems (referring to Kapoor et al., Gene 162: 175 (1995) (attached)), the two pieces of cDNA were ligated together and directly transcribed, and the mixture of transcription products introduced into cells. For Kunjin virus, one-piece infectious clones were obtained. Polo et al. 1997 succeeded because they used a non-conventional system. Polo et al. 1997 assembled full-length dengue virus type 2 cDNA in yeast.

Polo et al. 1997 teaches away from the claimed subgenomic replicons, because, rather than being the basis for extending the preparation of a stable full-length cDNA copy of dengue virus type 2 RNA capable of producing an infectious RNA transcript in vitro to prepare a dengue virus type 2 self-replicating RNA (subgenomic replicon) with a deletion in the region of the genome encoding structural components of the virion, it criticizes, discredits, and otherwise discourages the preparation of a stable full-length cDNA copy of dengue virus type 2 RNA capable of producing an infectious RNA transcript in vitro. This is because only a non-conventional system would work, namely, yeast. Thus, contrary to the position taken by the Patent Office analogizing to Kunjin virus, it would NOT have been obvious to extend Polo et al. 1997 to prepare a dengue virus type 2 self-replicating RNA (subgenomic replicon) with a deletion in the region of the genome encoding structural components of the virion.

For these reasons, the prior art would teach away because a person of ordinary skill in the art, upon reading the references, would be led in a direction divergent from the claimed subgenomic replicons; and perhaps towards solving the problem in the prior art of the need for

[1] This is not a concession that a prima facie case of obviousness has been established or that any claim limitations are taught or suggested by the prior art reference or references when combined.

one-piece infectious clones using a conventional expression system for the purpose of studying dengue RNA replication (the motivation proposed by the Patent Office). The conclusion is that the claims are non-obvious over the references. Thus, the claims are in compliance with 35 U.S.C. §103.

Claim 16 was rejected as being unpatentable over Khromykh & Westaway, J. Virol. 71: 1497 (1997), in view of Polo et al., J. Virol. 71: 5366 (1997) and Fields Virology 3rd ed., Philadelphia, Pa., Lippincott-Raven Publishers, pp. 931 et seq. (1996), optionally in view of Westaway et al. (USP 6,893,866, see also WO 99/28487 of which the USP is a continuation application). Fields is said to teach that flaviviruses (of which dengue is a species) have structurally similar genomes. Claims 18-20 were rejected as being unpatentable over Westaway et al. (USP 6,893,866, see also WO 99/28487 of which the USP is a continuation application), in view of Polo et al., J. Virol. 71: 5366 (1997), optionally in view of Khromykh & Westaway, J. Virol. 71: 1497 (1997). Nevertheless, teaching away is the very antithesis of § 103 obviousness. The additional references (Khromykh et al., J. Virol. 74: 3253 (2000), describing elements in the Kunjin genome, was optionally included in the above rejections for obviousness) and new combinations of references do not negate the fundamental teaching away of the prior art.

3. Compliance with Rules Against Double Patenting

The Patent Office provisionally rejected certain of the pending claims under the judicially created doctrine of obviousness-type double patenting as being unpatentable over selected claims of U.S. Pat. Appl. No. 11/192,923, filed July 29, 2005, or U.S. Pat. Appl. No. 11/194,342, filed still later based on its Serial No. The rule according to MPEP 804 I B 1 is that if a "provisional" obviousness-type double patenting rejection is the only rejection remaining in the earlier filed of the two pending applications, while the later-filed application is rejectable on other grounds, the examiner should withdraw that rejection and permit the earlier-filed application to issue as a patent without a terminal disclaimer. Where there are three applications containing claims that conflict such that an obviousness-type double patenting rejection is made in each application based upon the other two, it is not sufficient to file a terminal disclaimer in only one of the applications addressing the other two applications. Rather, an appropriate terminal disclaimer must be filed in at least two of the applications to link all three together. Here, a "provisional" obviousness-type double patenting rejection is the only rejection remaining in the earliest filed of

all three pending applications, because this application was filed September 5, 2003, while U.S. Pat. Appl. No. 11/192,923 was filed July 29, 2005 and U.S. Pat. Appl. No. 11/194,342 was filed even later based on its Serial No. Consequently, an appropriate terminal disclaimer can be filed in the two later-filed applications to link all three together if an obviousness-type double patenting rejection remains in the two later-filed applications. Thus, the examiner is respectfully requested to withdraw the rejection in this application and permit the earliest-filed application to issue as a patent without a terminal disclaimer.

4. Rescission and Retraction of Prior Traversal of Restriction Requirement

The claims of the present application were subject to a restriction requirement. In response to the restriction requirement, Applicant provisionally elected with traverse. Applicant hereby rescinds and retracts such traversal.

5. No Disclaimers or Disavowals

Although the present communication may include alterations to the application or claims, or characterizations of claim scope or referenced art, the Applicants are not conceding in this application that previously pending claims are not patentable over the cited references. Rather, any alterations or characterizations are being made to facilitate expeditious prosecution of this application. The Applicants reserve the right to pursue at a later date any previously pending or other broader or narrower claims that capture any subject matter supported by the present disclosure, including subject matter found to be specifically disclaimed herein or by any prior prosecution. Accordingly, reviewers of this or any parent, child or related prosecution history shall not reasonably infer that the Applicants have made any disclaimers or disavowals of any subject matter supported by the present application.

CONCLUSION

Applicant respectfully requests that a timely Notice of Allowance be issued in this case. If any points remain that can be resolved by telephone, the Examiner is invited to contact the undersigned at the below-given telephone number.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

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Completion of Kunjin Virus RNA Sequence and Recovery of an Infectious RNA Transcribed from Stably Cloned Full-Length cDNA

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Completion of the Kunjin virus (KUN) RNA sequence showed that it is the longest flavivirus sequence reported (11,022 bases), commencing with a 5′ noncoding region of 96 bases. The 3′ noncoding sequence of 624 nucleotides included a unique insertion sequence of 46 bases adjacent to the stop codon, but otherwise it had properties similar to those of RNAs of closely related flaviviruses. A full-length KUN cDNA clone which could be stably propagated in *Escherichia coli* DH5α was constructed; SP6 polymerase RNA transcripts from amplified cDNA were infectious when transfected into BHK-21 cells. A mutational change abolishing the *Bam*HI restriction site at position 4049, leading to a conservative amino acid change of Arg-175 to Lys in the NS2A protein, was introduced into the cDNA during construction and was retained in the recovered virus. Extra terminal nucleotides introduced during cloning of the cDNA were shown to be present in the in vitro RNA transcripts but absent in the RNA of recovered virus. Although recovered virus differed from the parental KUN by a smaller plaque phenotype and delayed growth rate in BHK-21 cells and mice, it was very similar as assessed by several other criteria, such as peak titer during growth in cells, infectivity titer in cells and in mice, rate of adsorption and penetration in cells, replication at 39°C, and neurovirulence after intraperitoneal injection in mice. The KUN stably cloned cDNA will provide a useful basis for future studies in defining and characterizing functional roles of all the gene products.

Flaviviruses are a family of small enveloped viruses with a capped single-stranded RNA genome of positive polarity, are transmitted by arthropods, and cause a range of diseases, including yellow fever, encephalitis, and dengue fever (55). In Australia, Murray Valley encephalitis and (rarely) Kunjin encephalitis occur sporadically in relatively small epidemics during summer, when the mosquito vector is abundant. Since the yellow fever virus (YF) RNA sequence was published in 1985 (38), genomes of at least 11 species including Kunjin virus (KUN) have been sequenced, in whole or in part. These results have consistently shown the presence of a single long open reading frame of over 10 kb coding for three structural and seven nonstructural (NS) proteins (18, 33, 40; for a review of earlier studies, see reference 7). In conjunction with N-terminal amino acid sequencing and carboxypeptidase digestion, the KUN data positively identified for the first time the four small NS proteins (NS2A, NS2B, NS4A, and NS4B), with their boundaries including the correct carboxy termini of NS1 and NS3, and a new consensus cleavage site Val-X-Ala (14, 41–43). Subsequently, we expressed virtually all of the KUN proteins, either singly or from contiguous coding sequences, by in vitro translation using RNA transcribed from cDNA (14a) or from recombinant vaccinia virus in cells (34).

A long-term goal is to identify functions of all the flavivirus NS proteins (13, 54). RNA polymerase and methyltransferase motifs have been reported to be present in NS5 (25, 38), and motifs for serine protease and helicase activity have been reported to be present in NS3 (19, 20). The only functions established to date are protease activity of the NS2B-NS3 complex (7–9, 10, 17, 36, 51) and nucleoside triphosphatase and triphosphatase activities of the carboxy-terminal region of NS3 (46, 49, 52, 53). Before proceeding to investigate these and other functional roles of KUN NS proteins, we decided to validate the quality of our cDNA clones by ligating them to produce stably cloned full-length cDNA of KUN RNA from which infectious RNA might be transcribed. Constructs for such transcription have been achieved for YF and Japanese encephalitis virus (JE) by ligating two cDNA fragments (37, 44) and for dengue type 4 virus by ligating and then amplifying full-length cDNA in *Escherichia coli* (27).

In this article we describe completion of the sequence of the untranslated regions (UTRs) of KUN RNA (not published previously) and preparation of stably cloned full-length cDNA. An infectious virus in which KUN RNA sequences were preserved but which had some distinct phenotypic properties was recovered.

MATERIALS AND METHODS

Cells and virus. Vero and BHK-21 cells were grown in M199 medium or in minimal essential medium (MEM), both supplemented with 5% fetal calf serum (FCS). Wild-type (WT) KUN strain MRM 61C was grown in Vero cells; purification of virus and extraction of genomic RNA were performed as described previously (14).

Completion of the nucleotide sequences in the 5′ and 3′ UTRs of the KUN RNA. Cloning and sequencing of the UTRs were completed by modification of the method used for tick-borne encephalitis virus RNA (29). Briefly, RNA was extracted from purified KUN grown in Vero cells, decapped with tobacco acid pyrophosphatase (Epicentre), and ligated with RNA ligase (Pharmacia) prior to being copied into cDNA across the junction region (i.e., of the 5′ and 3′ UTRs), by using reverse transcriptase and an 18-mer primer, 5′ GC CCGGGTCGGAGCAATT 3′, complementary to the sequence located 173 to 190 nucleotides (nt) after the initiation codon (14). This first-strand cDNA was purified and copied

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into double-stranded cDNA by the high-fidelity Pfu polymerase (Stratagene) in the PCR. The 777-bp product including nt 1 to 120 (5′) and nt 10366 to 11022 (3′) was blunt-end cloned into the *Sma*I site of *E. coli* plasmid pUC18. The nucleotide sequencing was performed by the dideoxy chain termination method (39).

Construction of the plasmid containing full-length cDNA of KUN. The clones from the original KUN cDNA library prepared by Coia et al. (14) and some larger clones prepared from them (14b) were used in construction of a full-length cDNA clone. The numbers represent the nucleotide positions in the completed full-length cDNA sequence of 11,022 nucleotides (see Fig. 2). First of all, an *Eco*RI^{3924}-*Bam*HI^{4806} fragment from pSPI14 was modified to eliminate an internal *Bam*HI^{4049} site by amplification using a primer which contained two mismatches with respect to the original cDNA sequence (G-4049→A and C-4054→T) but, otherwise, was complementary to the cDNA sequence at nt 4041 to 4062. The introduced substitution G-4049→A leads to a conservative amino acid change from Arg to Lys in NS2A protein (amino acid 175), whereas the substitution C-4054→T is corrected after *Bgl*II-*Bam*HI ligation of the cDNAs. The identity of the PCR-amplified sequence of 125 bp with the original cDNA sequence and the presence of the introduced mutation (G-4049→A) were confirmed by sequence analysis (data not shown). The plasmid containing the modified *Eco*RI^{3924}-*Bam*HI^{4806} fragment with the *Bam*HI^{4049} site removed was named pGRB*.

For the 5′ and 3′ UTRs, cDNA for each was obtained by PCR amplification from the plasmid containing the cDNA of the ligated 5′ and 3′ UTRs described above. The 5′-end primer for amplifying the 5′ UTR contained the SP6 promoter and the first 18 bases of the 5′ KUN sequence and was designed so that the first nucleotide of the KUN cDNA sequence would be adjacent to the SP6 promoter with just one extra base (G) between them (see Fig. 3). The recombination PCR technique was applied for subcloning of the 5′ UTR into a modified pGEM3Zf(+) vector (23). Briefly, the PCR product of the 5′ end containing the first 96 bases of the KUN sequence and sequences overlapping with the pGEM3Zf(+) vector were recombined in *E. coli* with the PCR-amplified pGEM3Zf(+) vector into which an *Mlu*I restriction site was introduced immediately upstream of the SP6 promoter. At the 3′ end of the cDNA of the genome an *Xho*I site was introduced during amplification of the complete 3′ UTR with appropriate primers. Fragments including the whole 5′ and 3′ UTR sequences from these cDNAs were ligated with the larger cDNA clones described below.

Two large subgenomic clones representing the entire genome, pBS5′H and pBS3′H, were initially prepared in the plasmid vector pBluescript (Stratagene) as follows. The pBS5′H clone containing the 5′ half of the genome from the beginning until the *Bam*HI^{4806} site was constructed by ligation of two fragments as shown in Fig. 2 into a *Bss*HII- and *Bam*HI-digested plasmid vector pBluescript II SK. The pBS3′H clone containing the 3′ half of the genome was constructed by ligation of three fragments as shown in Fig. 2 into a *Bam*HI- and *Xho*I-digested vector pBluescript II KS. The large fragments containing ligated KUN cDNA sequences from both pBS5′H and pBS3′H then were transferred into the plasmid vector pBR322 and were named pBR5′H and pBR3′H, respectively. Assembly of the final plasmid pAKUN containing full-length cDNA was completed by ligation of plasmid pBR5′H digested with *Pvu*I and *Bam*HI^{4806} (used as a vector) with the *Bam*HI^{4806}-*Pvu*I fragment from plasmid pBR3′H. *E. coli* DH5α was used for cloning and amplification of plasmid pAKUN.

In vitro transcription. Plasmid pAKUN containing full-length KUN cDNA was linearized by cleavage with *Xho*I and recovered by phenol-chloroform extraction and ethanol precipitation. The linearized DNA (2 μg) was added to a 50-μl reaction mixture containing 40 mM Tris (pH 7.5); 6 mM $MgCl_2$; 10 mM NaCl; 10 mM dithiothreitol; 50 U of RNasin; 2 mM spermidine; 1 mM each CTP, ATP, and UTP; 0.5 mM GTP; and 40 U of SP6 RNA polymerase (Promega). In order to incorporate a cap structure at the 5′ end of RNA transcripts, the synthetic cap analog $m^7G(5')ppp(5')G$ (New England Biolabs) at 1 mM was also added to the reaction mixture, which was incubated at 37°C for 2 h. The cDNA template was then digested with RQ DNase (Promega) for 20 min at 37°C.

RNA transfection. Three different transfection protocols were applied for recovery of infectious virus from in vitro-synthesized RNA transcripts using transfection reagent Lipofectin (GIBCO BRL) or Dotap (Boehringer Mannheim) and subconfluent monolayers of BHK-21 cells in six-well plates, prewashed with phosphate-buffered saline (PBS). In protocol 1, 10 μl of RNA transcripts (≈5 μg or less) and 8 μl of Lipofectin were each separately diluted in 150 μl of PBS, mixed, and allowed to incubate at room temperature for 15 min. The transfection mixtures were then transferred to cells for incubation at room temperature for 15 min. The inoculum was then replaced with MEM–2% FCS, and the cultures were incubated for 7 to 9 days at 37°C. In protocol 2, the amount of Lipofectin was increased to 20 μl; 10 μl of RNA transcripts (≈5 μg or less) and Lipofectin were each separately diluted in 750 μl of OPTI-MEM medium (GIBCO BRL), mixed, and incubated as in protocol 1, except that the transfection mixtures were left on cells for 6 h at 37°C before being replaced with MEM–2% FCS. Cells were further incubated for 3 to 5 days. In protocol 3, 10 μl of RNA transcripts (≈5 μg or less) and 15 μl of Dotap were each separately diluted in 50 μl of HBS buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.3], 150 mM NaCl), mixed, incubated at room temperature as before, and added to cells in 1 ml of MEM–2% FCS for 16 h at 37°C; the medium was then replaced with fresh MEM–2% FCS, and the cells were further incubated for 3 to 5 days.

Combined RNA transcribed from a pool of cDNA clones was transfected according to protocol 1; protocols 2 and 3 were used with RNA transcripts from individual cDNA clones.

Virulence of recombinant KUN in mice. Litters of 2- to 4-day-old BALB/c mice (six per litter) were inoculated intracerebrally with 20 μl of a 10-fold dilution of WT KUN or the recombinant AK virus. Dead mice were counted every day, and the 50% lethal dose (LD_{50}) titer was calculated.

Nucleotide sequence accession numbers. The 5′ and 3′ UTR sequences of KUN RNA have been deposited in GenBank and have been given accession numbers L24511 and L24512.

RESULTS

Definition and characterization of the 5′ and 3′ noncoding regions of WT KUN RNA. The nucleotide sequence was determined in both directions across the cDNA of the circularized KUN genomic RNA for three individual clones as described in Materials and Methods. The same sequence was obtained for each. The 5′ UTR comprised 96 bases, and the 3′ UTR comprised 624 bases (Fig. 1A and B). Sequences overlapping with the previously published KUN RNA sequence differed only at nt 10525 to 10529 (GAAGU rather than UAG [Fig. 1B]). The complete nucleotide sequence of KUN RNA is

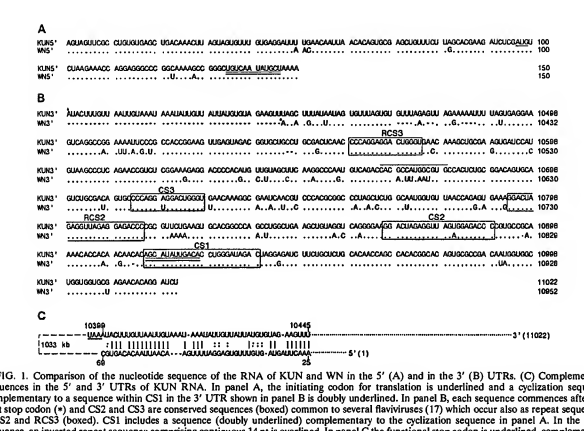

FIG. 1. Comparison of the nucleotide sequence of the RNA of KUN and WN in the 5′ (A) and in the 3′ (B) UTRs. (C) Complementary sequences in the 5′ and 3′ UTRs of KUN RNA. In panel A, the initiating codon for translation is underlined and a cyclization sequence complementary to a sequence within CS1 in the 3′ UTR shown in panel B is doubly underlined. In panel B, each sequence commences after the first stop codon (*) and CS2 and CS3 are conserved sequences (boxed) common to several flaviviruses (17) which occur also as repeat sequences, RCS2 and RCS3 (boxed). CS1 includes a sequence (doubly underlined) complementary to the cyclization sequence in panel A. In the KUN sequence, an inverted repeat sequence comprising contiguous 14 nt is overlined. In panel C the functional stop codon is underlined, complementary nucleotides are joined by vertical lines, and possible G · U pairs are indicated by colons. Gaps in alignment (dashes) and homologous nucleotides (dots) are indicated. The WN RNA sequences were published previously (6). The bounds of CS3 and RCS3 were determined by alignment of GenBank sequences for KUN, WN, JE and Murray Valley encephalitis virus.

now defined as 11,022 bases, the longest of all published flavivirus RNA sequences. Because of the strong homology (79%) between KUN and West Nile virus (WN) RNA sequences (6, 14, 50), careful comparisons were made of their UTRs. The 5′ UTRs were both 96 nt long, and there were only four differences (Fig. 1A). In contrast, the 3′ UTR of KUN RNA was 53 nt longer than that of WN RNA (Fig. 1B) and 42 nt longer than that of JE RNA, the next longest published sequence (582 nt [45]).

It was established (6, 21, 50) that the 3′ UTRs of WN, JE, and Murray Valley encephalitis virus include, in addition to the flavivirus conserved sequence CS1, two other conserved nucleotide sequences of 23 and 16 nt, which are repeated. These occur also in the KUN sequence (CS1, CS2 and RCS2, and CS3 and RCS3 in Fig. 1B). The CS1 sequence (21) is located at nt 10916 to 10939 (Fig. 1B), and in KUN and WN RNAs CS1 contains the 5′ AGCAUAUUGACA 3′ sequence (KUN nt 10916 to 10927), which is complementary to the sequence 5′ UGUCAAUAUGCU 3′ within the coding region of core protein (nt 135 to 146 [Fig. 1A]). The sequences within them, 5′ CAUAUUGA 3′ and 5′ UCAAUAUG 3′, respectively, are perfectly conserved among all flaviviruses; Hahn et al. (21) have proposed that these sequences could form a panhandle structure by cyclization of the flavivirus RNA molecule. Also within the KUN 3′ UTR there is a unique inverted repeat sequence, 5′ CACGCCAUGGCGUG 3′, at nt 10666 to 10679 which is absent from the 3′ UTR of WN and other flaviviruses.

The 3′-terminal 83 nt of KUN and WN RNAs (adjacent to CS1) differ by only 4 nt (Fig. 1B) and have the potential to form the same stem-loop structure described for several flaviviruses by others (4, 15, 21, 22, 30, 31, 38, 45, 47, 50), with ΔG values of about −60 kcal (ca. −250 kJ), rather larger than those reported for the equivalent structures in JE, YF, and dengue type 2 virus RNAs (21, 46) (ΔG, −38.6 to −41 kcal [ca. −162 to −170 kJ]).

The extra length of the KUN 3′ UTR arises almost entirely from a unique sequence of 46 nt commencing at nt 10399 immediately after the first stop codon; beyond this sequence there is very close homology (88%) to the WN 3′ UTR. When homologous or complementary sequences in the UTRs of KUN and other flavivirus RNAs were sought, perfect complementarity was observed between the 5′ UTR sequence 5′ AAACUU 3′ (nt 25 to 30) and the 3′ UTR sequence 3′ UUUGAA 5′ (nt 10445 to 10440) of KUN and between the 5′ UTR sequence 5′ ACAAUUAACA 3′ (nt 54 to 63) and the 3′ UTR sequence 3′ UGUUAAUUGU 5′ (nt 10414 to 10405) of KUN. Complementarity in the intervening regions within the 5′ and 3′ UTRs of KUN was less prominent (Fig. 1C), but overall there was 55% complementarity (or 70% including G · U pairing) between nt 10399 to 10445 (the unique sequence) and nt 69 to 25. The nt 25-to-30 and 54-to-63 sequences noted above are identical in KUN and WN RNAs, but no complementarity for these sequences was found within the WN RNA sequences. Thus, only in KUN RNA could a

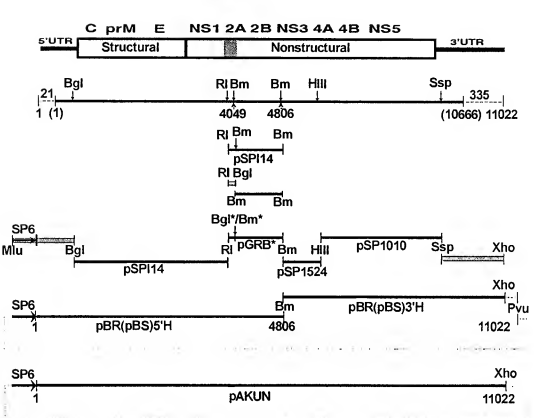

FIG. 2. Construction of plasmid pAKUN containing full-length cDNA of KUN RNA. The assembly of KUN full-length cDNA in the plasmid vector pBR322 was performed as described in Materials and Methods. The numbers in parentheses (1 and 10666) represent the first and the last nucleotide positions, respectively, in the previously published sequence (14), with the exception that position 10666 here corresponds to position 10664 in the published sequence because of a noted deletion of 2 nt in the 3' UTR. The numbers 21 and 335 represent the numbers of end terminal nucleotides which were not previously cloned and sequenced (14) (Fig. 1). The numbers 1 and 11022 represent the first and the last nucleotides, respectively, in the completed full-length cDNA sequence. Restriction sites: Bgl, *Bgl*I; Bm, *Bam*HI; RI, *Eco*RI; HIII, *Hind*III; Ssp, *Ssp*I; Xho, *Xho*I; Mlu, *Mlu*I; Pvu, *Pvu*I. SP6, RNA polymerase promoter. Shaded boxes, PCR-amplified cDNA fragments; solid lines, cDNA fragments derived from previously obtained plasmid clones; dashed lines, newly cloned cDNA; dotted lines, pBR322 sequence; asterisks, no-longer-existent restriction sites *Bgl*I and *Bam*HI. The loss of these sites during cloning is described in the text. Two contiguous segments of plasmid pSPI14 were used at different stages of the construction.

second panhandle structure be formed, by cyclization between complementary regions such as those defined above for the 5' and 3' UTRs.

We searched GenBank without success for viral RNA sequences with ≥30% homology (in plus sense and minus sense) to the KUN 46-nt sequence 10400 to 10445 (55% homology to nt 69 to 25, excluding G·U pairing). Only four DNA entries showed a region of similar homology for the KUN 46-base sequence: plasmid TiS4 (61%), herpesvirus saimiri (52%), yeast mitochondrial DNA (61%), and *Drosophila* genes *z600*, *gdl*, *Eip28/29*, and *max1* (74%). The terminal regions show very high homology between the KUN 5' and 3' UTRs (nt 10401 to 10414 [93%] and nt 10437 to 10445 [89%] [Fig. 1C]). When the four DNA sequences were compared with the same terminal regions, the relative homology ranged from 52 to 74%. There appears to be no compelling reason to assume a DNA origin of the 46-base insert in KUN RNA.

Construction of the pAKUN plasmid containing full-length KUN cDNA. The cDNA sequences of the 5' and 3' UTRs of KUN genomic RNA were incorporated into two large subgenomic clones pBS5'H and pBS3'H (Fig. 2) and amplified in the high-copy-number vector pBluescript, as described in Materials and Methods. Initial attempts to prepare a plasmid clone containing full-length cDNA using these plasmids were unsuccessful. A major difficulty was experienced in propagating the 5'-half subgenomic clone in the vector pBluescript (but not the 3'-half subgenomic clone). Transformed colonies appeared clear compared with the white color of normal DH5α vector colonies, and they grew more slowly. In liquid medium, no growth was apparent for 16 h after inoculation, and 30 to 40 h of total incubation was necessary for growing an adequate amount of bacterial culture. Purification of the recombinant plasmids by subsequent cloning did not change their phenotype. However, after the cDNA containing the 5'-half fragment from pBluescript was transferred into the pBR322 vector to obtain pBR5'H, the recombinant-containing colonies appeared to be normal and grew at the same rate as the colonies containing the parental vector. For assembly of the full-length cDNA the 3' half from pBS3'H was also recloned into the pBR322 vector as pBR3'H. After ligation of cDNA from pBR5'H and pBR3'H at the *Bam*HI^{4049} site, we recovered six individual full-length cDNA clones in pBR322 which were

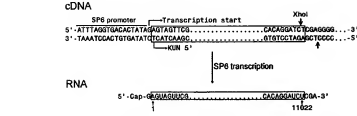

FIG. 3. 5′ and 3′ termini of the KUN cDNA template and in vitro-synthesized RNA. Plasmid pAKUN containing full-length cDNA was linearized by *Xho*I digestion before transcription by SP6 RNA polymerase to produce a runoff product. The resulting RNA should contain 1 extra nt (G) at the 5′ end and 3 extra nt (CGA) at the 3′ end. The sequences of cDNA and RNA corresponding to the authentic KUN sequences are boxed. Arrows in cDNA, *Xho*I restriction site; arrows in RNA, 5′- and 3′-terminal nucleotides corresponding to the authentic WT KUN RNA sequence.

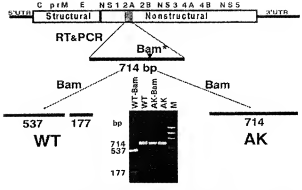

FIG. 4. Recombinant KUN (AK virus) contains the mutation G-4049→A introduced into the cDNA template. AK virus was recovered from BHK cells transfected with the RNA transcript of pAKUN DNA containing a mutation abolishing the *Bam*HI[4049] site, shown in the NS2A region. The recovered AK virus was then exhaustively treated with RNase A and used to infect BHK cells. The total cytoplasmic RNA from these cells and from WT KUN-infected cells was used for cDNA synthesis and PCR amplification with KUN-specific primers upstream and downstream of the NS2A gene. *Bam*HI digestion of this 714-bp PCR fragment yielded two fragments of 537 and 177 bp in the case of WT KUN infection but did not affect the size of the DNA fragment amplified from the RNA isolated from AK virus-infected cells, as expected. RT, reverse transcription.

stable and grew normally in *E. coli* DH5α, and their KUN origin was verified by restriction mapping (data not shown).

In vitro synthesis of infectious RNA and recovery of recombinant virus. Figure 3 shows the cDNA sequence at the 5′ and 3′ termini of KUN RNA, bounded by the SP6 promoter and the *Xho*I site used for preparing runoff transcripts. Note that the transcripts should contain 1 extra nt (G) at the 5′ end and 3 extra nt (CGA) at the 3′ end. In the initial experiment *Xho*I-digested cDNAs from four of the cDNA clones described above were combined for production of runoff RNA transcripts which were transfected with Lipofectin (protocol 1 in Materials and Methods). Cytopathic effects (CPE) were first observed on day 7 posttransfection, only in the cells transfected with the largest amount of RNA (≈5 μg). CPE then developed quickly, and tissue culture fluid was collected on day 8 posttransfection as a source of recovered virus. No signs of infection were observed in other cells transfected with 10-fold dilutions of the RNA by day 9 posttransfection. Recovered virus was passaged twice in BHK-21 cells in T-80 flasks, with 1 ml of harvested culture fluid being transferred at each passage. The virus titer reached 2.5×10^5 PFU/ml by 56 h postinfection (first passage) and 1.6×10^6 PFU/ml by 30 h postinfection (second passage). A titer of 6×10^6 PFU/ml was attained at 48 h after infection of BHK-21 cells with 0.01 PFU/cell virus material from the second passage per cell. This third passage pool was designated AK virus and was used in further characterization experiments.

In separate experiments individual RNAs transcribed from four of the cDNA clones were each used for transfection with Lipofectin or Dotap (protocol 2 or 3, respectively). Infectious virus was recovered from transcripts of each clone, with only slight differences in the time of appearance of CPE (3 to 5 days). In all transfections only the highest concentration of RNA (≈5 μg) produced CPE, with none being produced by any clone by day 5 posttransfection with 0.5 μg of RNA or less. In contrast, transfection of similar amounts of virion RNA from either WT KUN or AK virus using Lipofectin (protocol 1) resulted in severe CPE at the highest concentration (≈100 ng) and many plaques in culture under liquid medium at the lowest concentration (≈1 ng) by day 3 posttransfection, indicating that transfection efficiencies of purified viron RNA from WT and AK viruses were similar.

To prove that, in fact, a constructed full-length cDNA clone is stable after propagation in *E. coli* and, hence, could be used further in mutagenesis studies, we retransformed one of the clones (N7) in *E. coli*, picked four individual colonies, grew them in liquid growth medium, purified the plasmid DNAs, and used them as templates for producing in vitro RNA transcripts. Transfections of BHK-21 cells with all four RNAs using Dotap liposomes resulted in CPE with efficiencies that were similar to each other and to that for the original N7 clone.

Molecular characterization of recovered AK virus. In order to distinguish recombinant virus from parental WT KUN, we had introduced a mutation abolishing the *Bam*HI[4049] restriction site in the NS2A gene by PCR-directed mutagenesis in the cDNA template (see Materials and Methods) (Fig. 2). Total cellular RNAs isolated from BHK-21 cells infected with recovered viruses were analyzed for the presence of the above mutation in viral RNAs. Mock-infected and WT-virus-infected cells were used as negative and positive controls in the reactions, respectively. To prevent any possible contamination of virus pools with the input template RNA used for the original transfections, all viruses were exhaustively treated with RNase A prior to infection. Isolated total cellular RNAs were reverse transcribed with a minus-sense primer (nt 6452 to 6471). The resulting cDNAs were PCR amplified with a plus-sense primer (nt 3526 to 3541) and a minus-sense primer (nt 4202 to 4218), and the obtained PCR fragments were digested with *Bam*HI. As shown in Fig. 4, the *Bam*HI[4049] site was absent as expected in the cDNA derived from AK virus RNA. The same results were obtained for all the analyzed viruses recovered after transfection of RNA transcripts from individual cDNA clones (data not shown). The mutation G-4049→A, abolishing the *Bam*HI[4049] site, was also confirmed by sequencing analysis of AK virus-derived cDNA (data not shown).

To investigate whether the extra nucleotides added to the 5′ end and 3′ end of in vitro-synthesized RNA were retained or lost in the AK virion RNA, we sequenced the ends of both RNAs. Recovery and sequencing of the PCR fragment containing ligated 5′- and 3′-terminal sequences of cDNAs copied from in vitro-synthesized RNA (transfected RNA) and puri-

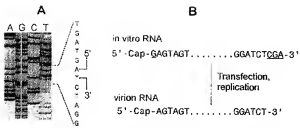

FIG. 5. Nucleotide sequences of the 5′ end and 3′ end of AK virus RNA. (A) Nucleotide sequence of PCR-amplified cDNA in the region surrounding the ligated 5′ and 3′ ends of AK virion RNA. The fragment containing cDNA of the ligated 5′ and 3′ ends was obtained as described in Materials and Methods. The last nucleotide at the 3′ end and the first nucleotide at the 5′ end of cDNA are indicated. (B) Expected sequence containing extra bases at the 5′ and 3′ ends of in vitro-synthesized RNA (underlined), which were absent in virion RNA of recovered progeny AK virus.

fied virion RNA were performed as described in Materials and Methods. As shown in Fig. 5, the sequences at the 5′ end and at the 3′ end of AK viral RNA were identical to those of parental (WT) viral RNA. Sequence analysis of 5′ and 3′ termini of vitro-transcribed RNA showed that extra bases including the added 5′ G were indeed present (data not shown). The sequences of the first 93 nt of the 5′ UTR and the last 78 nt of the 3′ UTR of AK viral RNA were also determined and proved to be identical to that of WT virus RNA (data not shown).

Biological characterization of recovered AK virus. Plaque assay revealed a smaller plaque phenotype of all of the recovered viruses including those derived from recloned cDNA compared with WT KUN (Fig. 6). The plaque morphology of AK virus seemed to be uniform and was not changed after four virus passages in BHK cells (data not shown), although plaques on BHK cells were rather larger than those on Vero cells (Fig. 6). The growth rates of WT and AK viruses were compared in BHK-21 cells infected at multiplicities of 0.01 PFU per cell. Growth of AK virus was delayed by several hours compared with that of the WT virus, and the AK virus titer was maintained in the culture for a longer period of time than that of the WT, possibly because of a slower CPE. Both viruses reached similar peak titers of infectivity, approximately 5×10^7 to 9×10^7 PFU/ml at 36 h (WT) and 48 h (AK) postinfection.

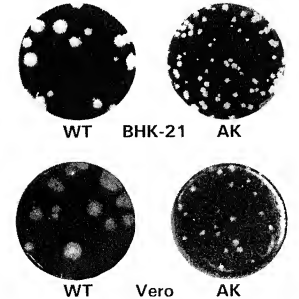

FIG. 6. Plaque morphology of parental (WT) KUN and recombinant AK virus in BHK-21 and Vero cells. Monolayers of BHK-21 or Vero cells were infected with WT KUN or AK virus, overlaid with agarose, and stained with crystal violet after 6 days (BHK-21) or 7 days (Vero) in culture.

To explore different factors which might cause the small plaque phenotype and delayed growth rate of AK virus, we first studied the possibility of the presence of a temperature-sensitive mutation in AK virus RNA. Plaque assays were carried out in Vero cells infected with AK virus or WT KUN, and cultures were incubated separately at 37 and 39°C. The titer of WT KUN was 2.5×10^7 at 37°C and 1×10^7 at 39°C. The corresponding titers of AK virus were 6×10^6 and 2.5×10^6, respectively. Thus, very small differences in virus titer at 37 and 39°C were observed in both AK or WT virus infections, showing that AK virus was not temperature sensitive relative to WT virus.

The rates of adsorption and penetration as possible causes of the observed phenotypic differences between AK and WT viruses were also compared. To compare the adsorption rates, monolayers of BHK-21 cells in six-well plates were infected in triplicate with ≈120 PFU of AK virus or WT KUN per well and the viral inoculum was allowed to adsorb for 30, 60, and 90 min at 37°C. Cells then were washed with PBS and overlaid with 1% agarose containing 5% FCS in MEM. Cells were cultured at 37°C for 3 to 4 days and stained with 0.2% crystal violet to visualize the plaques. The rate of adsorption was calculated as the ratio of average number of plaques at 30 or 60 min postinfection to the average number at 90 min postinfection, expressed as a percentage. In WT virus infection 36% of infectious virus particles (mean ratio, 60:168 PFU) were adsorbed at 30 min postinfection and 69% (115:168 PFU) were adsorbed at 60 min postinfection. In AK virus infection the corresponding percentages were similar: 37% (47:127 PFU) and 64% (81:127 PFU), respectively. To compare the rates of penetration, monolayers of BHK-21 cells in six-well plates were infected with ≈120 PFU of AK virus or WT KUN per well and the viral inoculum was allowed to adsorb for 30 and 90 min at 37°C. Then the cells were washed with PBS, and either hyperimmune anti-KUN or preimmune rabbit serum diluted 1/20 in MEM–1% FCS was added for another 60 min. After being washed with PBS, the cells were overlaid with 1% agarose, incubated, and stained as described above. The rate of penetration was calculated as the ratio between the average number of plaques in the wells treated with hyperimmune antiserum to the average number of plaques in the control wells treated with preimmune antiserum, expressed as a percentage. The ratio was 48% (47:97 PFU) for the WT virus infection and 60% (29:48 PFU) for the AK virus infection at 30 min postinfection, and 88% (224:256 PFU) and 85% (95:112 PFU), respectively, at 90 min postinfection. According to these results, no apparent differences were observed in the rates of adsorption or of penetration between WT and AK virus infections.

Pathogenicity of recovered AK virus in mice. The neurovirulence rates of the WT KUN and AK virus in suckling mice were compared after intracerebral and intraperitoneal inoculations (see Materials and Methods). The titer of WT KUN after intracerebral challenge was 2×10^6 LD_{50} per ml, and the

titer of AK virus was 5×10^7 LD_{50} per ml, which correlated well with their titers in Vero cells (2.5×10^7 and 6×10^6 LD_{50} per ml, respectively). The average survival time after intracerebral inoculation of 10 PFU (≈100 LD_{50}) was 4.5 days for WT virus and 6.0 days for AK virus. Intraperitoneal challenge of groups of 10 mice with 100 PFU (≈1,000 LD_{50}) resulted in 100% mortality for both viruses, but death was delayed for AK virus (7.2 days) compared with WT virus (5.2 days). Thus, a slight delay in mouse death was observed after infection by either route with AK virus compared with the WT KUN infection.

DISCUSSION

A stable full-length KUN cDNA clone was constructed, and from it infectious RNA was transcribed in vitro. The identity of recovered recombinant KUN (AK virus) and the presence of a mutation introduced into the cDNA used for preparing infectious RNA were confirmed by restriction enzyme digestion and sequencing analysis of the cDNA amplified from the total RNA of cells infected with AK virus. Sequencing analysis of the cDNA amplified from decapped and ligated AK virion RNA showed the absence of extra terminal nucleotides added during construction of the KUN cDNA clone and presumed to be present in the in vitro RNA transcript used for transfection (Fig. 4). To confirm this, we also sequenced the RNA transcripts by the same method and found that extra bases at the 5′ and 3′ termini were still present. These results provide the first direct demonstration of loss of extra terminal nucleotides during transfection and replication of in vitro-transcribed flavivirus RNA and are in accord with results reported for other RNA virus transcripts (reviewed in reference 1). However, the possibility that in every preparation of RNA transcribed from plasmid pAKUN a very small (undetectable) fraction lacked the extra terminal nucleotides and was infectious cannot be completely excluded.

This is the second report of a stably cloned full-length flavivirus cDNA propagated in a plasmid vector which resulted in transcription of infectious RNA, the other being achieved with dengue type 4 virus and chimeric viruses derived from it (2, 27, 35). As noted earlier, this was not possible with YF and JE (37, 44) presumably because of mutations introduced during propagation of ligated cDNA in *E. coli*. For example, transcripts from more than 100 independent full-length JE cDNA clones were not infectious (44). In our experiments infectivity was recovered after transcription from four distinct cDNA clones, and four more transformants cloned from one of these again yielded infectious RNAs. Although the efficiency of these transfected RNAs was much lower than that of virion RNA, the efficiency of transfection of RNA from recovered AK virus appeared to be similar to that of parental KUN. A number of authors (1, 27, 37, 44) have also noted the relatively low specific infectivity of RNA transcribed from cDNAs of other RNA positive-strand animal viruses. If only a small proportion of the transcripts represents RNA capable of replication, it would compete inefficiently with the majority of (defective) RNA. Alternatively, the presence of extra nucleotides at the 5′ end and 3′ end of in vitro-transcribed RNAs could also contribute to the initial low transfection efficiency compared with purified AK virion RNA, which did not contain these extra nucleotides.

The small plaque phenotype of recovered AK virus could be due to the introduced mutation leading to the change in amino acid 175 of NS2A from Arg to Lys. However, the substitution of Arg to Lys is a very conservative change and normally would not lead to any significant changes in protein structure and function. Comparison of the penetration and adsorption rates revealed no marked differences in these characteristics between AK virus and WT KUN, indicating that elements of the structural proteins involved in attachment and entry of the viral particles into the cells were not apparently changed. Small plaque mutants of KUN (isolated from persistent infections of mosquito cells) or JE (chemically induced) are temperature sensitive (16, 32), and temperature-sensitive mutants of Sindbis virus and of poliovirus contain mutations in the RNA polymerase genes (5, 48). However, no significant reduction of AK virus titer was observed during incubation at 39°C compared with that at 37°C. Sequence analysis of the first 93 and the last 77 nt of AK virus genomic RNA which form a highly conserved stem-loop structure in the 5′ and 3′ UTRs, respectively, revealed no difference from the sequence of WT KUN RNA. Since these sequences are considered likely to be involved in recognition of the RNA template by the RNA replication complex (3, 4, 11, 12, 38), the data suggest that this step of viral replication was also not significantly changed for recombinant AK virus. In summary, we have excluded several possible causes of the small plaque phenotype and of the slightly delayed growth rate of the recombinant AK virus. It is possible that a minor change in sequence such as the introduced mutation in the NS2A gene may cause delay in RNA replication rate or delay in assembly and release of virus particles, but we cannot be precise at present.

We searched all other published flavivirus sequences for evidence of a sequence similar to the KUN 3′ UTR sequence nt 10399 to 10445, which appears to have been inserted immediately downstream from the stop codon (Fig. 1B), and found a vestige of it in the first 19 nt of the 3′ UTR of Murray Valley encephalitis virus RNA (28), which have 60% homology with KUN nt 10427 to 10444. It is theoretically possible that the RNA polymerase may at some remote time in the past have temporarily switched copying from the 3′ region of an ancestor KUN RNA (plus) template in the vicinity of the stop codon to the 3′ UTR of an adjacent RNA (minus) strand and later returned to copying from the 3′ region of the original RNA (plus) template, still in the vicinity of the stop codon (nt 10396 to 10398). An insert produced by copy choice (24) in the proposed manner would be inverted, as shown in Fig. 1C. An unreleased ribosome or ribosomal subunit on the stop codon may have blocked the path of the polymerase during one of its early traverses of the RNA (plus) template, facilitating the proposed switching. Interestingly, the homology in coding of KUN and WN has been preserved upstream of the stop codon; the terminal six amino acids at the carboxy terminus of NS5 for both viruses are identical: Val-Glu-Asp-Thr-Val-Leu (14). The significance of the apparent 46-base insertion remains uncertain; more sequence data are required for additional flavivirus strains.

Although the major KUN-specified proteins E, NS1, NS3, and NS5 have been expressed from cDNA in recombinant vaccinia viruses and identified antigenically or by size by gel electrophoresis (26, 34), these results provided no information on the functional ability of these proteins in virus replication or assembly. The recovery of infectious virus from RNA transcripts based on the same cDNA used in preparing the recombinant vaccinia viruses has established the authenticity of these KUN products and, indeed, of all the noncoding and coding content of KUN cDNA. It will now be possible to use stably cloned full-length cDNA capable of producing an infectious RNA transcript in further mutagenesis studies to define functional roles of KUN proteins in viral replication, assembly, and maturation.

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Synthesis and characterization of an infectious dengue virus type-2 RNA genome (New Guinea C strain)

(Recombinant DNA; positive-strand RNA virus; flavivirus; T7 RNA polymerase; in vitro transcription; RNA transfection; site-directed mutagenesis; reverse transcriptase-based PCR)

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SUMMARY

Dengue virus type 2 (DEN-2), a member of the Flaviviridae family, has a positive-strand RNA genome, 10 723 nucleotides (nt) in length and encoding a single polyprotein precursor consisting of 3391 amino acids (aa). In order to construct a full-length cDNA clone, the viral genome was cloned into 5′ (nt 1–2203 under the control of the T7 promoter (p_{T7})) and 3′ (nt 2203–10 723) constructs. A full-length DEN-2 cDNA under pT7 control was assembled in vitro after excising the two cDNA inserts from the 5′ and 3′ constructs, and joining them with T4 DNA ligase. The RNA produced by in vitro transcription of the cDNA using T7 RNA polymerase was infectious, as shown by transfection of permissive BHK-21 and Vero cells, and propagation of the virus particles released into the culture media. The virus particles stably maintained the conservative mutation introduced into the 5′ construct, and the cells infected with the infectious RNA-derived virus synthesized virus-specific DEN-2 antigens, as shown by immunofluorescence and immunoprecipitations. The full-length infectious clone for DEN-2 should be useful for the study of molecular mechanisms involved in viral RNA replication and virus assembly.

INTRODUCTION

The four serotypes of dengue virus (DEN) have the widest geographic distribution and highest morbidity among the more than 70 members of the family Flaviviridae (Westaway et al., 1985; Monath, 1994). The virus is transmitted by *Aedes aegypti* mosquitos, and infection in humans could lead to a simple manifestation of the disease, dengue fever and occasionally more severe forms, dengue hemorrhagic fever and dengue shock syndrome, especially among children (Halstead, 1988). The molecular basis of pathogenesis by dengue virus is least understood.

DEN-2 contains a single-stranded RNA genome of

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Abbreviations: aa, amino acid(s); BHK, baby hamster kidney; bp, base pair(s); C, capsid protein; DEN, dengue virus; DMEM, Dulbecco's modified Eagle's medium; E, envelope protein; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; HMAF, hyperimmune mouse ascetic fluid against a mixture of DEN-2 antigens; IC, infectious clone; kb, kilobase(s) or 1000 bp; NS, non-structural protein(s); nt, nucleotide(s); ORF, open reading frame; PAGE, polyacrylamide-gel electrophoresis; PBS, phosphate-buffered saline (10 mM Na·phosphate pH 7.2/150 mM NaCl); PCR, polymerase chain reaction; pfu, plaque-forming unit(s); PMSF, phenylmethylsulfonyl fluoride; Polk, Klenow (large) fragment of *E. coli* DNA polymerase I; p_{T7}, T7 promoter; RT, reverse transcription; SDS, sodium dodecyl sulfate; *Taq* polymerase, DNA polymerase isolated from *Thermus aquaticus* YT1; *UTR*, untranslated region(s); vTF 7-3, recombinant vaccinia virus expressing T7 RNA polymerase; wt, wild type; YF, yellow fever virus.

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positive-strand polarity consisting of 10 723 nt (in New Guinea-C strain; Irie et al., 1989) and a type-I cap at the 5′ end, but devoid of a poly(A) tail at the 3′ end (Rice et al., 1985; 1986; for a review, see Chambers et al., 1990a). The viral RNA contains 5′ and 3′ noncoding regions, 96 and 454 nt in length, respectively, and a single long ORF which encodes a polyprotein of 3391 aa. The N-terminal region, encoding the structural components of the virion, C (capsid), prM (precursor membrane) and E (envelope), is processed by host signal peptidase associated with the endoplasmic reticulum (ER) (Svitkin et al., 1984; Markoff, 1989; Nowak et al., 1989; Ruiz-Linares et al., 1989). The viral NS2B/NS3 protease having the specificity for dibasic aa is involved in the processing of precursor for the non-structural proteins NS1–NS5 (see Bazan and Fletterick, 1989; Gorbalenya et al., 1989; Chambers et al., 1990b; 1991; Preugschat et al., 1990; Falgout et al., 1991; Wengler et al., 1991; Cahour et al., 1992; Zhang et al., 1992; Falgout et al., 1993). The map order is 5′-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3′.

Primary sequences of several flavivirus genomes are available (for a review, see Chambers et al., 1990a, and references therein). Beginning with poliovirus genome more than a decade ago (Racaniello and Baltimore, 1981; Van der Werf et al., 1986), functional full-length cDNA clones have been constructed for many positive-strand RNA viruses and have provided valuable tools for studying the molecular mechanisms involved in viral genome replication and virion assembly. Full-length cDNA clones of a few flavivirus genomes have also been constructed (YF, Rice et al., 1989; DEN-4, Lai et al., 1991; Japanese encephalitis virus, Sumiyoshi et al., 1992; Kunjin virus, Khromykh and Westaway, 1994), as well as subgenomic long expression constructs for DEN-2 (Zhang and Padmanabhan, 1993; Yamshchikov and Compans, 1993). The aim of the present study is to construct a full-length cDNA clone for DEN-2 (New Guinea-C strain) from which infectious RNA transcripts could be generated in vitro which will be useful for studying the mechanism of RNA replication and virion morphogenesis.

RESULTS AND DISCUSSION

(a) Cloning of 5′ and 3′ constructs

Our initial attempts to clone the full-length cDNA for DEN-2 into low-copy number plasmids such as pLG-SPORT and pBR322 failed due to the inherent genetic instability of such plasmids in *E. coli* especially when the 5′ portion of DEN-2 was present. Thus, for construction of full-length cDNA, we cloned the genome in two fragments which could be joined in vitro to form a full-length template and used for transcription of infectious RNA (Fig. 1). The 5′ portion (nt 1–2203) was cloned into pUC18 vector (5′-Construct in Fig. 1) and the rest of DEN-2 genome was cloned into pBR322 (3′-Construct in Fig. 1). The 5′-construct contains the viral genome sequences encoding the 5′-*UTR*, C, prM and E proteins (missing only 22 aa), and the 3′-construct encodes the C-terminal region of E (which includes 22 aa), NS1–NS5 and the 3′-*UTR*. The two constructs have a common *Bam*HI site at nt 2203. Whereas the complete digestion of the 3′ construct with *Bam*HI + *Xba*I could give rise to a DNA fragment encompassing nt 2203–10 723 of the DEN-2 genome, the DNA fragment containing the *p*T7 and nt 1–2203 was obtained in poor yields because the

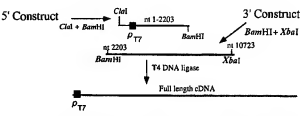

Fig. 1. Strategy for construction of infectious clone (IC). **3′ construct:** Step 1: In this study, the clone pPMM158 containing 3133 bp between *Stu*I (nt 7590) and *Xba*I (nt 10723) of the DEN-2 genome in pGEM-7Zf* vector was first constructed from the cDNA clones pPM-F-12 and pPM-PCR1 (Irie et al., 1989). A *Bam*HI-*Xba*I fragment (nt 7590–10 723) from pPMM158 was cloned into a low-copy-number vector pLG-SPORT to yield pMK8. Step 2: A 5-kb *Eco*RV fragment (nt 4322–9333) obtained from pLZ-12345 plasmid (Zhang and Padmanabhan, 1993) was cloned into the *Eco*RV site of pBR322 vector to yield pMK5 clone. Step 3: *Stu*I (nt 7590)-*Sal*I (present in the vector portion of pMK8 clone downstream from *Xba*I) fragment was inserted in the place of *Stu*I (nt 7590)-*Sal*I (downstream from *Eco*RV at nt 9333) fragment in the pMK5 clone, to yield the clone pMK6.4 (contains nt 4322–10 723). Step 4: A 3.1-kb fragment obtained by *Cla*I (a site in the pBR322 vector upstream to *Bam*HI (at nt 2203 in the clone pLZ-12345; Zhang and Padmanabhan, 1993) and *Nsi*I (nt 4696) digestion of pLZ-12345 was inserted in the place of the fragment between *Cla*I (upstream to the 5′ boundary at nt 4322) and *Nsi*I (at nt 4696) of pMK6.4 clone to give rise to pMK8.5 (which contains nt 2203–10 723 of DEN-2). **5′ construct:** Step 1: A cDNA clone was constructed in which the 5′-*UTR* was flanking the P_{T7} and the chloramphenicol acetyl transferase (CAT) gene in pUC18 vector (pLZ-5′UTR/CAT) (L.Z. and R.P., unpublished results). This clone was digested with *Xba*I (upstream to the P_{T7}) + *Bgl*II (a site in the 5′-*UTR*) and a 100-bp fragment was inserted into the pKT2.4 cDNA (Irie et al., 1989) digested with *Xba*I + *Bgl*II. Steps 2 and 3: The resulting clone, pMK9 (nt 1–2333 placed downstream from P_{T7}), containing three *Bam*HI sites (upstream to P_{T7} (site 1), at nt 1696 (site 2) and at nt 2203 (site 3)) was modified to destroy site 1 by partial digestion, fill-in by Pollk, ligation and cloning, which created a *Cla*I site. Then, site 2 was destroyed by PCR using primer # 1 containing a mutation in the *Bam*HI site (GGATCC→GGATC**A**, which will not change the aa sequence) and the wt primer # 2 and pMK9 clone as the template. The blunt-ended PCR product was cloned into pMK9 plasmid to yield pMK10 (5′ construct). The 5′-construct was digested with *Cla*I + *Bam*HI and the 3′-construct was digested with *Bam*HI + *Xba*I, and the 2.2-kb and 8.5-kb DNA fragments were purified from these digests. The two fragments were ligated in vitro, using T4 DNA ligase to give rise to the full-length cDNA.

5'-construct initially had three *Bam*HI sites, one just upstream from $p_{T7\ (site\ 1)}$ and two at nt 1696 and 2203 (sites 2 and 3). In order to obtain the 5' portion of DEN-2 cDNA in sufficient yield, the *Bam*HI sites 1 and 2 were destroyed as detailed in the legend to Fig. 1. Site 1 was first destroyed which resulted in the generation of a *Cla*I site upstream to the p_{T7}. The *Bam*HI site at nt 1696 was destroyed by PCR by introducing a conservative point mutation in the coding sequence for E protein. The 5'-construct was then digested by *Cla*I + *Bam*HI and the 2203-bp DNA fragment was purified. The 3'-construct was digested with *Bam*HI + *Xba*I and the DNA fragment encoding the rest of DEN-2 cDNA was isolated.

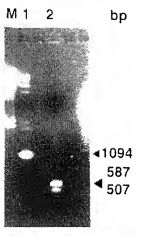

Fig. 2. Synthesis of infectious DEN-2 RNA from the in vitro joined cDNA templates and stable propagation of virus derived from RNA transfection. The ligated full-length DNA as described in the legend to Fig. 1 was used as template for in vitro transcription in a reaction mixture (100 µl) containing 40 mM Tris (pH 7.5)/6 mM $MgCl_2$/10 mM NaCl/10 mM dithiothreitol/50 units of RNasin/2 mM spermidine/1 mM each CTP, ATP, UTP, CTP and m7G(5')ppp(5')G (New England Biolabs)/50 units of T7 RNA polymerase (Promega, Madison, WI, USA). After 2 h of incubation at 37°C, the cDNA template was digested with RNase-free DNase (Promega) for 15 min at 37°C. Subconfluent monolayers of BHK-21 and Vero cells in 6-well (35 mm diameter) cell culture plates were washed with OPTI-MEM (GIBCO-BRL) and incubated with 500 µl of OPTI-MEM containing 20 µl of Lipofectin™ and 5 µl of RNA from in vitro transcription reaction (5 µg or less) for 6 h at 37°C before being replaced by DMEM containing 10% serum. After 7 days of incubation, medium was collected and used as source of infectious clone-derived virus (IC DEN-2) for titering the infectivity and for infecting fresh BHK 21 and Vero cells. The medium collected after 7 days was used for infectivity assay by ELISA (Trirawatanapong et al., 1992) and for infection of fresh BHK-21 cells, followed by [^{35}S]methionine labeling and immunoprecipitation analysis of DEN-2-specific antigens. To examine the stability of IC DEN-2 during propagation, BHK-21 cells were infected with either wt DEN-2 or IC DEN-2, and the medium was collected after 7 days. Total RNA was isolated using standard procedures. 1 µg of the RNA was used for the reverse transcriptase reaction using the Ampli-Taq RT-PCR kit (Perkin-Elmer) according to the manufacturer's instructions in a reaction containing 5 µl of the cDNA mixture and two PCR primers, 5'-TCTAAGGAAGTACTGTATA (nt 1100–1119) and 5'-TCCT-CCCAGGGATCCAAAATC (nt 2196–2217). The PCR products were digested with *Bam*HI and analyzed by 1% agarose-gel electrophoresis. Lanes: 1, λ DNA digested with *Hin*dIII; 2 and 3, *Bam*HI-digested cDNAs derived from IC DEN-2- and wt DEN-2- infected cells, respectively.

(b) In vitro transcription and transfection of full-length DEN-2 RNA

The 5'-*Cla*I-*Bam*HI fragment containing the T7 promoter was mixed with the 3' *Bam*HI-*Xba*I fragment in a molar ratio of 1:5 to ensure that there was no free 5'-fragment or its self-ligation product(s) present in the ligation reaction which could potentially interfere by serving as templates for in vitro transcription. The ligation mixture was digested with *Xba*I and was used for in vitro transcription catalyzed by T7 RNA polymerase. An aliquot was analyzed by formaldehyde-agarose gel electrophoresis, and it was estimated that 70–80% of the transcript was of full length (data not shown). The in vitro transcription reaction mixture was treated with RNase-free DNase I to digest the DNA templates prior to transfections of BHK-21 and Vero cells. 6 h after transfection, cells were incubated with DMEM containing 10% serum. After 7 days of incubation, an aliquot of the medium was saved for estimation of infectivity. Another aliquot was used to infect fresh BHK-21 and Vero cells, and the medium collected 7 days post-infection (second passage IC virus) was used for infectivity assay.

(c) Infectivity assay of the cloned DEN-2 virus

The infectivity titer of the DEN-2 virus produced from the transfections of BHK-21 cells with the full-length RNA was determined using the culture media collected from the first and second passages. The wt DEN-2 of known titer was used as control. Vero cells were grown to 70% confluency in 96-well plates, and infected with serially diluted wt DEN-2 of known titer as control or with the IC DEN-2 virus. Each serially diluted virus sample was used to infect cells in 16 wells. The expression of DEN-2-specific antigens was quantitated by first reacting with the polyclonal DEN-specific mouse hyperimmune serum, and then with a rabbit anti-mouse IgG conjugated to alkaline phosphatase. The activity of alkaline phosphatase was measured by ELISA as described (Trirawatanapong et al., 1992). A standard plot of absorbance vs viral titer was constructed for wt DEN-2-infected cells, and the titer for IC DEN-2 virus was estimated using this plot. The specific infectivity of the IC DEN-2 virus in the first passage medium was calculated to be 8.8×10^2 pfu/µg RNA transfected. The titer of the IC DEN-2 virus released in the first and second passage media is 3.5×10^3 and 2.8×10^4 pfu/ml, respectively (data not shown). The standard deviation was calculated from the absorbance data to be $\pm 0.2\%$.

(d) Analysis by RT-PCR of IC DEN-2 RNA for the loss of the *Bam*HI site at nt 1696

In order to verify that the virus particles of IC DEN-2 released from permissive cells were indeed derived from the transfected in vitro transcript, we analyzed the genomic RNA extracted from the virus particles by RT-PCR. The absence of *Bam*HI site at nt 1696 in the cDNA of IC DEN-2 RNA would indicate that the progeny virus particles are derived from IC DEN-2 and not from the wt DEN-2. Genomic RNAs from IC DEN-2 and wt DEN-2 were isolated from infected cells, and used for cDNA synthesis followed by PCR to amplify the sequences between nt 1109 and 2203. PCR products were then digested with *Bam*HI, and analyzed by agarose-gel electrophoresis (Fig. 2). As shown in Fig. 2, the cDNA sequence derived from the wt DEN-2 RNA was cleaved into 587 and 580-bp fragments as expected (lane 2), whereas that of IC DEN-2 was not digested at nt 1696 and, thus, only a single 1094-bp fragment was produced (lane 1). Moreover, since the in vitro IC DEN-2 transcript was digested by RNase-free DNase I prior to transfections, these results confirmed that IC DEN-2 is derived from the transfected RNA which retained the conservative point mutation introduced into the 5′ construct, and not from the carryover of template DNA (see section **a**).

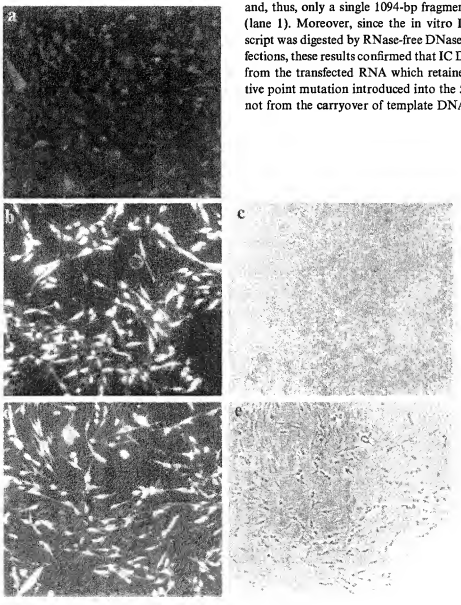

Fig. 3. Synthesis of virus-specific antigens in IC DEN-2- and wt DEN-2-infected cells analyzed by indirect immunofluorescence. BHK 21 cells were grown on coverslips placed inside Petri dishes, and infected with wt DEN-2 or IC DEN-2. Four days post-infection, cells were washed with cold PBS and fixed with acetone for 20 min. Cells were incubated for 2 h at room temperature with PBS containing 3% BSA and hyperimmune serum (1:50 dilution), washed 3 times with cold PBS for 10 min each and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG at a dilution of 1:50 in PBS containing 3% BSA for 45 min. Cells were then washed as before. A drop of 95% glycerol was added and the cells were visualized for fluorescence using a Nikon Diaphot microscope with epifluorescence attachment. Panels **a, b and d**: uninfected, wt DEN-2- and IC DEN-2-infected BHK-21 cells, respectively. Panels **c** and **e**: phase contrast pictures of cells in panels **b** and **d**, respectively.

(e) Synthesis of DEN-2 antigens in cells infected with IC DEN-2 and wt DEN-2 analyzed by indirect immunofluorescence and immunoprecipitations

The virus particles released from cells infected with IC DEN-2 or wt DEN-2 were analyzed for the synthesis of virus-specific antigens using the polyclonal hyperimmune serum by indirect immunofluorescence method. As shown in Fig. 3, both wt DEN-2-infected (Fig. 3b) and IC DEN-2-infected (Fig. 3d) BHK-21 cells showed positive immunofluorescence as compared with the background fluorescence observed with the uninfected cells (Fig. 3a). Fig. 3c and e are the corresponding phase contrast pictures of monolayers shown at the lefthand side. Similar patterns were observed with Vero cells infected with either virus (data not shown).

Next, we examined whether the IC DEN-2-infected cells produced DEN-2 antigens in a similar way as the wt DEN-2-infected cells. Vero and BHK-21 cell monolayers were infected with IC DEN-2 or wt DEN-2. At four days postinfection, cells were labeled with [^{35}S]methionine and cell extracts were used for immunoprecipitation with polyclonal hyperimmune serum, which had antibodies against several DEN-2 antigens. The immunoprecipitates were analyzed by SDS-PAGE (Fig. 4). Similar patterns of viral antigens were synthesized in Vero and BHK-21 cells infected with either wt DEN-2 (lanes 3 and 4, respectively) or by IC DEN-2 (lanes 5 and 6, respectively), which were not produced in uninfected cells (lanes 1and 2). Low levels of E protein were produced both in wt DEN-2- and IC DEN-2-infected Vero cells, compared with BHK-21 cells (lanes 4 and 6).

We also examined the synthesis and secretion of NS1 in the cells infected with either IC DEN-2 or wt DEN-2. NS1 is a glycosylated protein and exists in multiple forms such as cell-associated, cell-surface (Schelesinger et al., 1985) and secreted dimeric forms (Schlesinger et al., 1985; Smith and Wright, 1985; Winkler et al., 1988; 1989; Mason, 1989; Lee et al., 1989). Since accurate processing and secretion of NS1 into the medium is an indication of authenticity of IC DEN-2, the media from BHK-21 and Vero cells infected with both viruses were examined. From the immunoprecipitation data, NS1 was found to be secreted into the culture media from IC and wt DEN-2-infected BHK-21 cells and Vero cells but not from uninfected cells (data not shown).

(f) Conclusions

Thus, the results of the experiments described above indicate that the IC DEN-2 RNA is infectious, as viable virus particles are released from transfected cells, which can then be amplified and used for infecting permissive cells. The viral RNA produced in the progeny virus particles stably maintain the conservative mutation that was introduced to destroy a *Bam*HI site on the cDNA. The infected cells are also shown to produce virus-specific antigens. Finally, full-length in vitro ligated DEN-2 cDNA, which can be transcribed to yield infectious RNA, will prove useful in mutagenesis studies to better understand the functions of various viral proteins in viral RNA replication.

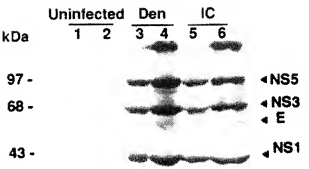

Fig. 4. Synthesis of virus specific antigens in IC DEN-2- and wt DEN-2-infected cells analyzed by immunoprecipitations. BHK 21 and Vero cells were grown to 70% confluency and infected with wt DEN-2 (1 pfu/cell), IC virus or mock-infected for 2 h at room temprature. The infected cells were incubated for 4 days and were washed with methionine-free medium (Sigma) supplemented with 2.5% dialyzed fetal bovine serum and incubated for 1 h to deplete endogenous methionine. [^{35}S]Methionine (ICN; 50 μCi/ml) was then added to a fresh medium with dialyzed serum. Labeled cells were incubated for i h in a lysis buffer (10 mM Tris·HCl pH 7.5/0.4 M NaCl/0.1% NP-40/1 mM PMSF. Cell extract (100 μl) was mixed with 100 μl of immunoprecipitation buffer (0.75% Triton X-100/0.7% NP-40/50 mM EDTA/2 mM PMSF), 10 μl preimmune serum and 30 μl of protein A-agarose suspension, and the mixture was incubated for 2 h at 4°C. The supernatant obtained by centrifugation was incubated overnight with mouse polyclonal hyperimmune serum (1:30) dilution, and 30 μl suspension of protein A-agarose beads. The immunoprecipitates were washed with a buffer containing 250 mM NaCl/50 mM Tris·Cl pH 7.4/5 mM EDTA/0.1% Triton-X100/0.5% NP-40/0.5 mM PMSF, followed by 0.1% SDS-6% PAGE and fluorography. Lanes: 1, 3 and 5, extract from infected Vero cells; 2, 4 and 6, extract from infected BHK-21 cells; 1 and 2, extract from uninfected cells; 3 and 4, wt DEN-2-infected cells (Den); 5 and 6, IC DEN-2-infected cells (IC).

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